Carbon Monoxide Analysis in Biological Specimens

1 Introduction

Carbon monoxide (CO) is a colorless, odorless gas produced by the incomplete combustion of organic fuels. It is also a common trace pollutant in the atmosphere. CO is present in the body at low concentrations, where it is bound to hemoglobin (Hb), to form carboxyhemoglobin (COHb). Normal COHb concentrations in the body are typically under 5% in nonsmokers, and up to 9% in smokers. COHb values below 10% are usually considered normal in healthy human subjects. Serious toxicity is often associated with COHb levels above 25%, and the risk of fatality is high with levels over 70%.

When CO is inhaled, it competes with oxygen (O_2) for the hemoglobin in red blood cells. The affinity of CO for hemoglobin is approximately 250 times that of O_2. When COHb is formed, O_2 cannot be transported to the tissues that need it, putting those tissues in a state of anemic hypoxia. Overexposure to CO produces headache, tremor, nausea, weakness, confusion, stupor and coma. Carbon monoxide poisoning may occur in fire victims or as a result of inhalation of automobile exhaust, heating system/stove waste products or other combustion gases.

2 Scope

This procedure quickly screens and quantitates biological specimens, typically whole blood, for elevated levels of COHb through a spectrophotometric method. Confirmation is completed by headspace gas chromatography with thermal conductivity detection.

3 Principle

Blood samples are screened and quantitated by spectrophotometry using a CO-Oximeter.

Specimens that screen positive by the spectrophotometric method are confirmed by gas chromatography with thermal conductivity detection (GC/TCD). Sulfuric acid is added to liberate the CO from the hemoglobin. An automated headspace sampler then samples and injects a portion of the headspace onto the GC/TCD system. The %COHb can be estimated using the amount of Hb detected by the co-oximeter.

4 Specimens

The screening procedure method requires approximately 0.2 mL of whole blood. The GC/TCD confirmation procedure requires 0.33 mL of whole blood. Spleen or other blood-rich organs can be analyzed by these procedures.

5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

a. AVOXimeter® 4000 Whole Blood CO-Oximeter

b. Disposable Test Cuvettes for AVOXimeter® 4000 (Purchased from ITC, Edison, NJ)

c. Disposable syringes (1 mL)

d. Kimwipes and/or blood block pads

e. Vortex mixer

f. Saponin

g. Concentrated sulfuric acid (Reagent Grade)

h. Sulfuric Acid Solution (1M)

i. 20-mL headspace vials (HSV) with appropriate crimper/decrimper, magnetic crimp caps and septa

j. Calibrated pipettors (5 µL - 1000 µL volume capable)

k. Gas chromatograph (Agilent 6890N or equivalent) equipped with thermal conductivity detector, analytical column (J&W HP-Molesieve 30 m x 0.32 mm x 12.00 µm or equivalent) and headspace autosampler (Gerstel MPS2 or equivalent)

6 Standards and Controls

a. Formic acid (~89%, reagent grade)

b. 0.05 M formic acid solution (GC/TCD performance check):

Dilute 215 µL of formic acid to 100 mL with deionized water in a graduated cylinder or flask. Mix well and store in glass at room temperature. Stable for at least one year.

c. AVOXimeter optical quality control filters (supplied with the instrument)

d. Blood COHb Controls:
RNA Medical QC 253 Full Range CO-Oximeter Control, or equivalent.
Purchased from RNA Medical, Division of Bionostics, Inc.,
Available in three levels, as described in Table 1[1]:

Table 1: Three Levels of RNA Medical CO-Oximeter Controls

Level / Lot	total Hb, g/dL Average Value	total Hb, g/dL Range	COHb, % Average Value	COHb, % Range
1 / 34814	8.3	7.6-9.0	5.7	1.7-9.7
2 / 34913	13.7	12.6-14.7	16.1	11.6-20.6
3 / 35013	17.2	15.9-18.5	44.2	38.9-49.5

Since the Level 1 control contains COHb at a value less than 10%, it is considered to be a Negative Control. This Negative Control will be analyzed with each CO-Oximeter assay and with each TCD assay.

The Level 2 and Level 3 controls are considered Positive Controls, as they contain COHb at a level greater than 10%. Each Positive Control will be analyzed with each CO-Oximeter assay and with each TCD assay.

7 Calibration

Calibration is performed automatically in the AVOXimeter. The only variables that may be changed are the pathlength (which is supplied with each lot of cuvettes) and a value for Hüfner's number (1.39 is typically used). These values do need not to be changed unless a new lot of cuvettes is used. See the AVOXimeter Manual for guidance.

8 Sampling

Not applicable.

[1] Average and range values provided by RNA Medical package insert. Consult appropriate package insert for target values, lot numbers, stability and storage.

9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

9.1 Screening and Quantitating %COHb by CO-Oximeter

9.1.1 Daily CO-Oximeter Checks

a. Turn the AVOXimeter on and wait for the "---READY--- Insert Cuvette" message to appear.

b. Insert the yellow optical quality control filter.

c. At the "Select Sample Type" screen, type "2/Enter" for QC.

d. At the "Select QC Type" screen, type "2/Enter" for Optical.

e. At the "Select Filter" screen, type "1/Enter" for Yellow. Hit "Enter" for OK if prompted.

f. The results will appear within ten seconds. Press "Print" to print a copy. (Sometimes "Print" must be pressed twice.)

g. Verify that the results are within the specifications on the sticker on the filter. Record whether the results Pass or Fail in the instrument logbook. (Target value ranges are printed on each filter.)

h. Repeat steps b. through g. for the orange filter. (This time, type "2/Enter" in step e. for the orange filter.)

9.1.2 Analyzing Samples and Controls by CO-Oximeter

a. Verify that the "---READY--- Insert Cuvette" message appears.

b. After mixing the blood sample via inversion, draw approximately 0.2 mL of a sample or control into a disposable syringe.

c. Insert the syringe into the cuvette.

d. Hold the syringe and cuvette at a 45 degree angle and gently press the plunger. Stop pressure when the sample reached the vent patch. (Do not allow vent patch to bulge.)

e. Verify that the light path area is free of bubbles.

f. Clean any drops of blood off of the exterior of the cuvette with a Kimwipe or blood block pad. If blood has broken through the vent patch, discard the cuvette and prepare a new one.

g. Insert the cuvette (with syringe still attached) into the AVOXimeter.

h. At the "Select Sample Type" screen, type "1/Enter" for Patient.

i. The results will appear within ten seconds. Press "Print" to print a copy. (Sometimes "Print" must be pressed twice.) Label the printouts with the correct sample name or lot number. (Note: printouts may be photocopied for inclusion in case notes since printer tape is not easy to read after a few months.)

j. After each sample is analyzed, the cuvette should be disposed of in biohazard waste.

k. Analyze all case samples and control(s) in duplicate, using a fresh cuvette each time.

9.2 Confirmation of %COHb by GC/TCD

The confirmation of elevated %COHb (defined as >10%) in a sample is analyzed using a headspace gas chromatographic method with detection by a thermal conductivity detector (GC/TCD). A liberating agent is added to affect the release of CO into the headspace. After an equilibration time, the samples are analyzed by GC/TCD.

9.2.1 Carbon Monoxide Testmix Analysis

a. To a 20 mL HSV, add 1 mL of concentrated sulfuric acid.

b. Add 50 µL of a 0.05 M formic acid solution.

c. Immediately crimp-seal the HSV and vortex for 10 seconds.

d. Incubate HSV at 100°C for 60 minutes in a laboratory heating block or a GC oven. (CO is produced quantitatively from the dehydration of formic acid in sulfuric acid.)

e. Analyze the headspace as per the instrumental conditions provided in Section 10 of this procedure.

f. Verify that the Decision Criteria for the Testmix defined in Section 11.1 of this procedure are met before continuing.

9.2.2 Analysis of Controls and Case Samples

a. For each control and case sample to be analyzed, label a clean 20 mL HSV with the sample name.

b. Using an adjustable pipettor, aliquot 0.33 mL portions of the appropriate blood sample or control into each HSV.

c. Add 0.33 mL liberating agent (1 M sulfuric acid) to HSV, immediately sealing each vial with a crimp cap.

d. Uniformly vortex each HSV for 30 seconds using a moderate setting. Avoid excessive splashing of sample onto crimp cap.

e. Analyze each HSV using the GC/TCD using the instrumental parameters in Section 10 of this procedure.

10 Instrumental Conditions

Confirmation of %COHb by GC/TCD

10.1 Gerstel MPS2 Headspace Sampler Parameters

syringe size:	1.0 mL (HS)	shake time (on/off):	30 / 2 s
syringe temperature:	70°C	injection volume:	900 µL
flush time:	2 min	injection speed:	900 µL/s
incubation temperature:	50°C	number of fill strokes:	5
incubation time:	20 min	GC cycle time:	5.3 min
shake speed:	250 rpm	PrepAhead:	enabled

10.2 Gas Chromatograph Parameters

Oven Parameters		Column Parameters		Inlet and Carrier Parameters	
temperature	40°C	type	HP-Molesieve	inlet temp.	250°C
isothermal		length	30 m	injection mode	split
run time	5 min	internal diameter	0.32 mm	carrier gas	helium
equilibration time	0.2 min	film thickness	12 µm	carrier mode	constant flow

Thermal Conductivity Detector Parameters				carrier flow	5.0 mL/min
temperature	205°C	makeup gas	helium	split ratio	3:1
reference flow	20 mL/min	makeup flow	2.5 mL/min		

11 Decision Criteria

11.1 CO-Oximeter

11.1.1 Daily Checks

Results from both optical filters should be within the manufacturer's specification ranges. If they are not, contact the instrument manufacturer for assistance.

11.1.2 Blood Controls by CO-Oximeter

The Negative Controls and the Positive Controls should all give COHb values within the manufacturer's specifications.

11.1.3 Unknown Samples by CO-Oximeter

Samples are considered "Negative" if the %COHb level is below 10%. Such samples will be reported as "None detected above a reporting limit of 10% COHb".

Samples are considered "Positive" if the %COHb level is above 10%. For these samples, the duplicate runs must be within 10% of each other. The average value for this test will be reported if the TCD confirmation results are also positive.

11.2 GC-TCD

11.2.1 Testmix Decision Criteria

The CO peak should be well separated from the nitrogen and oxygen peaks (>0.5 min baseline separation), and have a peak area greater than 200 units.

The GC column used in this procedure is a molecular sieve column, which may retain water. The column may be reconditioned by heating the GC oven to 225°C for >4 hours or overnight. Insufficient column conditioning results in poor chromatographic separation between the CO and air peaks.

11.2.2 Blood Controls by TCD

A detectable CO peak will be obtained from each Positive Control as well as from the Negative Control. Using the calculations described in Section 12, the Level 1 and Level 3 controls should calculate to within 5% (absolute) of the target value for Level 1 and within 20% (relative) of the target value for Level 3.

11.2.3 Unknown Samples by TCD

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the retention time of the peak should be within ±2% of the retention time obtained from injection of the testmix or positive control.

Using the calculations described in Section 12, the unknown sample results should agree within 20% (relative) of the average value obtained from the CO-Oximeter.

12 Calculations

%COHb is estimated from the TCD CO peak and the Hb amount measured by the CO-Oximeter as described in the example below:

1. Assume that the Level 2 Control Target Value (from the insert) is accurate. (For the example below, this value is 18.6%.)

2. Assume that the Hb average amount for each sample calculated by the AVOXimeter is correct.

3. Normalize the CO response for the unknown and the Level 2 Control.

a. $= \frac{\text{Unknown sample CO response (TCD area counts)}}{\text{Unknown sample tHb (from AVOXimeter)}}$

$= \frac{2036623}{16.6}$

$= 122688$

b. $= \frac{\text{Level 2 CO response (TCD area counts)}}{\text{Level 2 tHb (from AVOXimeter)}}$

$$= \frac{654049}{13.6}$$

$$= 48091$$

4. Use the normalized response for the unknown, as the normalized response for the Level 2 Control, and the target value for the Level 2 control to solve for the %COHb in the unknown.

$$\frac{122688}{x} = \frac{48091}{18.6}$$

$$x = 47.45\% \text{ COHb}$$

13 Uncertainty of Measurement

Two control levels (purchased from RNA Medical) were analyzed in triplicate over five days during the validation period. The "true" value of the controls was taken from the package insert. Our testing showed an average 2.7% positive bias for the two levels, and gave an overall standard deviation value of 1.3%. Since there is no certified reference material that we can base our calibration on, we must account for both the bias and the precision of these measurements to estimate our uncertainty. Therefore, these values will be summed to achieve a value of 4%. This value will be used as the historical uncertainty for the method, rather than the 1.3% value. Any positive case specimens will therefore be reported with a +/-12% uncertainty (relative) at a 99.7% confidence level. (For example, COHb was identified at a concentration of 50% ± 6% COHb, 99.7% CL, k=3.396).

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

14 Limitations

a. Bias of %COHb measurement (AVOXimeter; based on 15 measurements at each level on 5 days):

+4.17% (Low)
+2.90% (Medium)
+2.57% (Low)

b. Repeatability of %COHb measurement (AVOXimeter; based on 15 measurements at each level on 5 days):
   5.42% (Low)
   1.26% (Medium)
   0.96% (High)

c. Intermediate Precision of %COHb measurement (AVOXimeter; based on 15 measurements at each level on 5 days):
   8.62% (Low)
   1.33% (Medium)
   1.16% (High)

d. Reportable Range for %COHb (AVOXimeter): 10 - 75%

e. Other considerations:

   1. Samples to be analyzed should be rich in red blood cells.

   2. Serum-separated or "spun-down" blood samples are not appropriate for CO analysis.

   3. Samples that do not give acceptable results by the AVOXimeter may be considered unsuitable for %COHb measurement.

15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

16 References

AVOXimeter® 4000 Whole Blood CO-Oximeter Operator's Manual.

Lewis, R.; Johnson, R.; Canfield, D. *J Anal Tox*, 2004, *28*, 59-62.

Agilent Technologies, Publication A15836. "6890 Checkout Procedure TCD (Thermal Conductivity Detector)."

Agilent Technologies, Application Note. "Analysis of Permanent Gases and Methane with the Agilent 6820 Gas Chromatograph."

Kunsman, G.; Presses, C.; Rodriguez, P. *J Anal Tox*, 2000, *24*, 572-577.

Widdop, B. *Ann Clin Biochem* 2002, *39*, 378-391.

Lee, C.W. et al. *For Sci Inter*, 2003, *23*, 153-156.

Kunsman, G.W.; Levine, B., in *Principles of Forensic Toxicology*, 2nd Ed.; Levine, B., Ed.; AACC Press: Washington DC, 2003; Chapter 20.

Guidelines for Toxicological Quantitations (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Preparation of Chemical Reagents (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Safety Manual.

Instrument Subunit SOP Manual; FBI Laboratory Chemistry Unit.

Rev. #	Issue Date	History
0	06/21/06	New document that replaces a previous document also titled "*Carbon Monoxide Analysis in Whole Blood Samples*".
1	10/15/07	Updated title and section 2 to include other types of biological samples. Section 1 updated to reflect current literature. Updated procedure for Gerstel autosampler including instrumental parameters. Sections 6 and 7 revised to eliminate the use of calibrators. In section 9.1, increased amount of sodium dithionite and clarified its use. In section 9.2.2, added suggestion to analyze controls and samples in duplicate. Deleted 9.2.2p and re-lettered section. Updated sections 11, 13 and 14 based on historical use of this procedure.
2	03/05/10	Updated section 5 to allow for the use of disposable UV cuvettes and updated UV-Vis instrument model. Updated section 6 to include prepared in-house GC-TCD controls and description of UV-Vis versus GC-TCD controls. Updated section 9 to include use of disposable UV cuvettes. Updated UV-Vis software, removed Microsoft Excel reference in Section 10. Updated criteria for evaluation of UV-Vis and GC-TCD controls in section 11. Updated the calculations to include directions for automatic calculations using the instrument software in section 12. In section 14 updated limitations to differentiate between UV-Vis and GC-TCD parameters.
3	04/11/13	Replaced spectrophotometry screen with CO-Oximeter analysis and updated all affected sections. Changed TCD analysis from quantitative to qualitative and updated all affected sections. In 9.2.1.d, added option to heat in a GC oven.

Approval

Redacted - Signatures on File

Appendix 1: Abbreviated version of the CO Procedure for bench use.

Redacted - Form on File